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Exhaled markers of inflammation

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Exhaled markers of inflammation allow completely noninvasive monitoring of inflammation and oxidative stress in the respiratory tract in inflammatory lung diseases, including asthma, chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis and interstitial lung diseases. Such noninvasive techniques are simple to perform, may be repeated frequently and can be applied in children, including neonates and patients with severe disease in whom more invasive procedures are not possible. Several volatile chemicals can be measured in the breath (nitric oxide, carbon monoxide, hydrocarbons), and many nonvolatile molecules (mediators, oxidation and nitration products, proteins) may be measured in exhaled breath condensate.

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Abbreviations

CF	cystic fibrosis
CO	carbon monoxide
COPD	chronic obstructive pulmonary disease
NO	nitric oxide
NOS	nitric oxide synthase
PCD	primary ciliary dyskinesia

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Introduction

Many lung diseases involve chronic inflammation and oxidative stress. However, these are not measured directly in routine clinical practice because of the difficulties in monitoring inflammation. Several gases, such as nitric oxide (NO), carbon monoxide (CO), hydrocarbons, and nonvolatile markers and mediators (isoprostanes, leukotrienes, prostaglandins, cytokines, products of lipid peroxidation, nitrite/nitrate, S-nitrosothiols, nitrotyrosine) have recently been measured in exhaled air and condensate in adults and children. There is no single test that can be used to quantify airway inflammation. Peripheral blood markers are unlikely to be adequate, because the most important mediator and cellular responses occur locally within airways. Eosinophils in induced sputum originate from more proximal rather than small airways. It is clear that various markers of airway inflammation should be considered together to monitor asthma [1•]. Assessment of the 'exhaled breath profile' of several markers that reflect airway inflammation and oxidative stress, and are measured in exhaled air and condensate, is a promising approach in monitoring and management of patients with asthma and chronic obstructive pulmonary disease (COPD).

Exhaled gases

Outcome measures of airway inflammation and oxidative stress are needed for use in clinical practice. Exhaled breath analysis has been extensively used in the research rather than in the clinical setting to quantify airway inflammation and anti-inflammatory treatment in adults and children. We have shown that changes in, for example, exhaled nitric oxide (eNO) more accurately reflect clinical changes than single measurements. Repeated routine exhaled analysis may improve the clinical control of asthma, COPD, cystic fibrosis (CF), bronchiectasis and other lung diseases.

Nitric oxide

Exhaled NO is a useful and practical noninvasive marker that is related to airway inflammation [2,3], and elevated NO levels [4] are linked to polymorphisms in the nitric oxide synthase (NOS)1 gene and to asthma in Caucasian populations [5,6,7•]. Exhaled NO measurements have been validated against invasive measurements of inflammation by bronchoscopy and induced sputum [8], and can be made reproducibly [9] and therefore are comparable between different centres.

Increased levels of exhaled NO have been widely documented in patients with asthma [4,10], but not in

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patients with chronic cough [11], and may be useful in differentiating asthma from other causes of chronic cough. Exhaled and nasal NO may be used to identify individuals with atopy, because nonatopic asthmatic persons have normal exhaled NO [12•], and both adults and children with atopic asthma have higher levels of exhaled NO [13,14], which are associated with the magnitude of skin test reactivity [15], total immunoglobulin E [16] and blood eosinophilia [17]. Exhaled NO levels in stable COPD [18–20] are lower than in both smoking and nonsmoking asthmatic persons [21], and are not different from those in normal persons. Elevated NO levels in unstable COPD [22] may be explained by increased neutrophilic inflammation, oxidant/antioxidant imbalance, acidosis [23•] and sputum eosinophilia in some patients [24,25]. Pulmonary hypertension has the opposite effect, because COPD patients with cor pulmonale have low exhaled NO levels [26•], perhaps reflecting impaired endothelial NO release.

Because of the noninvasive character and practicality of exhaled and nasal NO measurements, they may be used cost-effectively for screening large populations, and as a useful biomarker of individual exposure to air pollutants [27–29]. An elevated exhaled NO may be found in patients with 'subclinical' forms of asthma (normal lung function, negative bronchodilator tests and elevated sputum eosinophilic cationic protein concentrations), as has been demonstrated in a study of over 8000 adolescent persons in Norway [30••].

It is most likely that exhaled NO is related to asthma control rather than to asthma severity [1•], and that serial NO measurements in individual patient over time may be useful to identify those patients who require a change in therapy. Exhaled NO has been used to monitor the effect of anti-inflammatory treatment in asthma [31] and asthma exacerbations [32,33•], because it is reduced by steroids, but is less affected by leukotriene antagonists [34–36]. A dose-dependent reduction in exhaled NO and improvement in symptoms has been shown in asthma [37], whereas the reduction in sputum eosinophils and similar improvement in symptoms was observed only after the higher dose of steroids [38•]. This suggests that exhaled NO may be too sensitive to determine whether inflammation is adequately controlled [1•]. Neither short-acting [31,39–41] nor long-acting [39,41,42] β_2-agonists reduce exhaled NO. Inhibition of NOS by NOS inhibitors [43,44], or inhaled prostaglandin E_2 [45] may be important in management of severe steroid-resistant asthma. Ibuprofen, a cyclo-oxygenase inhibitor, reduces the elevated levels of exhaled NO in normal individuals after endotoxin challenge [46], and indomethacin partly prevents an increase in exhaled NO and asthma symptoms in patients whose dose of steroids was reduced [47]. Low doses of theophylline have no effect on exhaled NO in asthma [48]. The immunosuppressive drugs cyclosporin and rapamycin inhibit NOS2 expression [49], suggesting that exhaled NO can be used to monitor the effect of these drugs.

Low exhaled and nasal NO, due to a deficiency in NOS2 [50], may be of diagnostic importance in cystic fibrosis (CF; Fig. 1) [51,52••]. Although there is a trend toward both exhaled and nasal NO being higher in patients who are not homozygous for the ΔF508 CF transmembrane regulator mutation [53•], there is no strong association between exhaled NO and disease severity in CF, or infection with *Pseudomonas* spp. [52••,54]. Exhaled and nasal NO are 'diagnostically low' in primary ciliary dyskinesia (PCD), a genetic disease that is characterized by defective motility of cilia [51,55,56]. Measurement of exhaled NO could be used as a screening procedure to detect PCD among patients with recurrent chest infections or male infertility due to immotile spermatozoa, and the diagnosis of PCD is then confirmed by the saccharine test, nasal NO, ciliary beat frequency and electron microscopy [57••]. Low levels of exhaled and nasal NO in PCD may be improved with L-arginine treatment [51].

Elevated nasal NO has been reported in allergic and perennial rhinitis, which is ameliorated by treatment with nasal corticosteroids [58]. However, increased expression of inducible NOS in nasal mucosa is not necessarily associated with a higher 3-nitrotyrosine-labelling intensity [59], suggesting that inducible NOS-derived NO may have a role in the pathophysiology of rhinitis, but the production of peroxynitrite in patients with rhinitis is not dependent on the level of rhinitis NOS alone [60].

Carbon monoxide

CO is a product of heme degradation by heme oxygenase, and reflects oxidative stress. Exhaled CO can be measured by electrochemical selective sensors in adults, children and neonates. Despite the early reports on elevated levels of exhaled CO in mild stable asthma [61], we have confirmed significantly elevated CO levels only in patients with severe asthma [62,63]; this possibly reflects high levels of oxidative stress and predominantly neutrophilic inflammation in these patients. The effect of inhaled steroids on exhaled CO in mild asthma is negligible [64]. In view of the simplicity of CO measurements and the portability of CO analysers, exhaled CO may be useful in noninvasive monitoring of paediatric asthma. For example, children with persistent asthma have significantly higher levels of exhaled CO than do those with infrequent episodic asthma [65•].

We have found elevated exhaled CO in ex-smoking COPD patients, suggesting ongoing oxidative stress or

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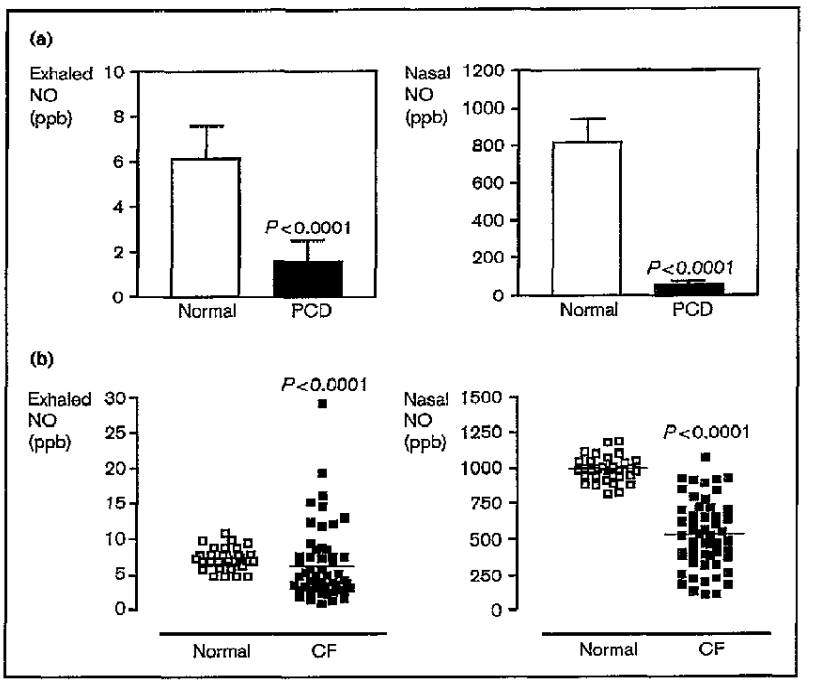
Figure 1. Exhaled and nasal nitric oxide

Exhaled and nasal nitric oxide (NO) in (a) primary ciliary dyskinesia (PCD) and (b) cystic fibrosis (CF). ppb, parts per billion. Adapted from Loukides *et al.* [51] and Thomas *et al.* [52••], respectively.



inflammation, but a major limitation of exhaled CO measurements in COPD is cigarette smoking, which masks any increase that may occur due to the disease process. In contrast to NO, exhaled CO levels were markedly elevated in stable CF patients [66•,67,68•], were increased further during exacerbations and were reduced with antibacterial treatment (Fig. 2) [53•]. This suggests that exhaled CO is not only a marker of oxidative stress/inflammation in CF, but also is a marker of disease severity, which is further confirmed by the finding of lower CO levels in patients on oral corticosteroid treatment [66•,67,68•]. We have shown [66•] that patients who are homozygous for the CF transmembrane regulator ΔF508 mutation have higher exhaled CO levels than heterozygous patients.

Hydrocarbons

Exhaled hydrocarbons, which are nonspecific markers of lipid peroxidation, may help to estimate the magnitude of in-vivo lipid peroxidation and to monitor the effect of novel drugs with antioxidant properties. Exhaled ethane levels are elevated in asthma [69•], COPD [70•] and CF [68•] (Fig. 3). The measurements of two different exhaled markers, for example NO and pentane, might be helpful to distinguish severe nocturnal asthma from obstructive sleep apnea, which is associated with low levels of circulating nitrite/nitrate [71]. Patients with CF have elevated levels of exhaled ethane, which is significantly correlated with exhaled CO and airway obstruction, supporting the view that oxidative stress and lipid peroxidation are increased in the airways of patients with CF.

Exhaled condensate

Exhaled breath condensate is collected by cooling or freezing of exhaled air, and the first studies to identify surface-active properties, including pulmonary surfactant, of exhaled condensate were reported in Russia during the 1980s [72,73]. Recently, several inflammatory mediators, oxidants and ions have been identified in exhaled breath condensates. Abnormalities in condensate chemistry and exhaled markers reflect intrinsic abnormalities of the airway lining fluid caused by inflammation and oxidative stress, and may be a valuable means of monitoring of lung diseases. The collection requires 10–15 min of tidal breathing to obtain 1–3 ml condensate, and is well tolerated by patients with severe airway obstruction and by children (Fig. 4a). Exhaled condensate is analysed by gas chromatography or extraction spectrophotemtry, or by different immunoassays (e.g. enzyme-linked immunosorbent assay).

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Figure 2. Exhaled carbon dioxide

Exhaled carbon dioxide (CO) in cystic fibrosis (CF): (a) disease severity and (b) effect of antimicrobial treatment. ppb, parts per billion. Adapted from Antuni *et al.* [53•].

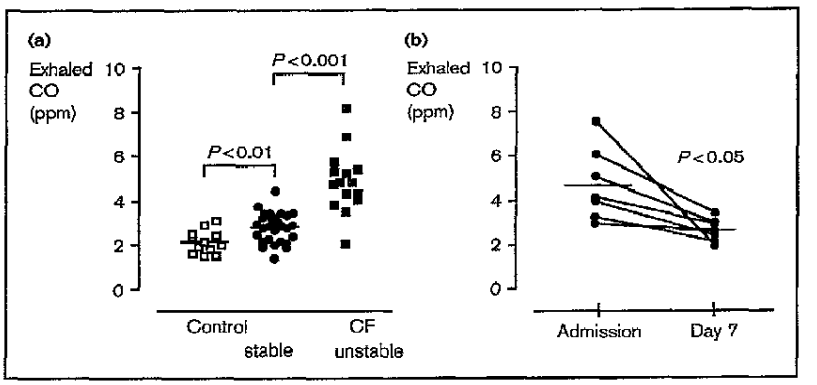
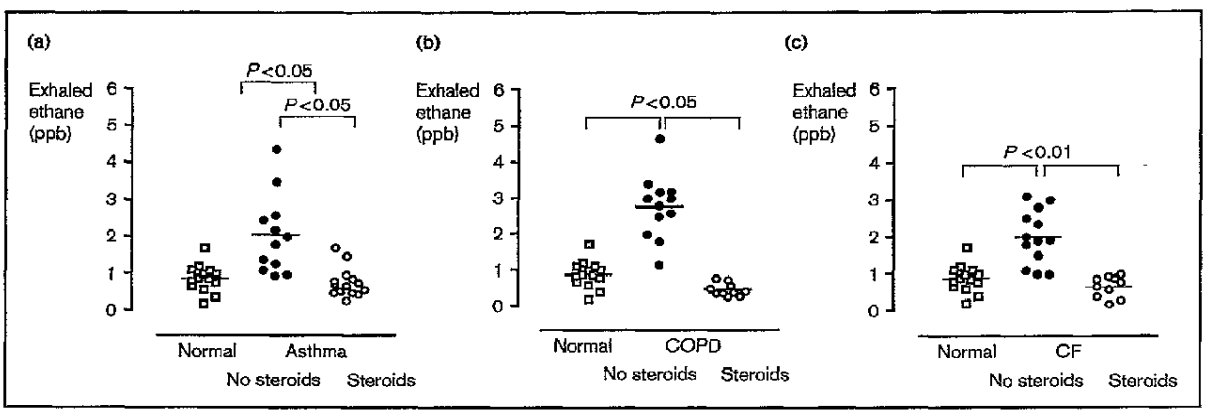


Figure 3. Exhaled ethane



Exhaled ethane in (a) asthma, (b) chronic obstructive pulmonary disease (COPD) and (c) cystic fibrosis (CF). ppb, parts per billion. Adapted from Paredi *et al.* [69•], Paredi *et al.* [70•] and Paredi *et al.* [68•], respectively.

Isoprostanes and prostaglandins

Isoprostanes (non-cyclo-oxygenase-derived prostaglandin products of arachidonic acid) can be detected in exhaled breath condensate by enzyme-linked immunosorbent assay, which is comparable to gas chromatography coupled with mass spectrometry (GC/MS) analysis. They reflect cellular effects of oxidative stress, and 8-isoprostane levels were doubled in mild asthma as compared with in normal persons, and were increased by threefold in severe asthma [74•,75] (Fig. 4b). Exhaled 8-isoprostanes are increased in CF [67], normal cigarette smokers and to a much greater extent in COPD patients [76•]. Lack of an effect of corticosteroids on 8-isoprostane is due to their ineffectiveness at inhibiting oxidative stress [37•]. There is increased expression of inducible cyclo-oxygenase (cyclo-oxygenase-2), which forms prostaglandins and thromboxane in asthma and COPD [77]. Indeed, exhaled prostaglandin E_2 and $F_{2\alpha}$ are markedly increased in patients with COPD, but not in asthma [75].

Leukotrienes

Exhaled leukotriene B_4, C_4, D_4, E_4 and F_4 are increased in asthma (Fig. 4c) [78••], during the late asthmatic response to allergen challenge [79] or after steroid withdrawal in moderate asthma. The latter increase is related to elevated exhaled NO and worsening of asthma symptoms [79]. This may suggest that exhaled nitrotyrosine may predict the asthma deterioration caused by inflammation [78••]. Leukotriene B_4 concentrations are

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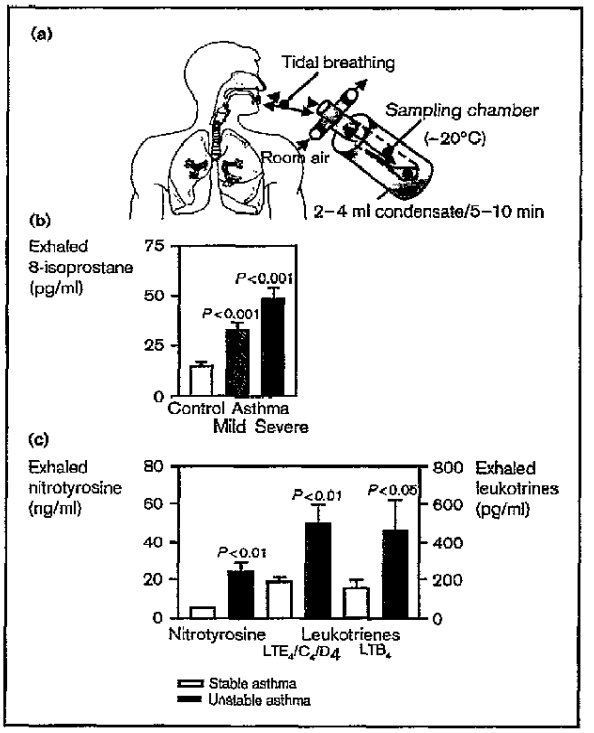
Figure 4. Exhaled breath condensate



(a) Diagram of the apparatus. (b) Exhaled 8-isoprostane in asthma; adapted from Montuschi *et al.* [74•]. (c) Exhaled nitrotyrosine and leukotrienes (LTs) before and after steroid withdrawal in patients with moderate asthma; adapted from Hanazawa *et al.* [78••].

increased in exhaled breath condensate of patients with COPD and in moderate and severe asthma [78••]. This suggests that leukotriene B_4 may be involved in exacerbations of asthma and may contribute towards neutrophil recruitment.

Nitrate, nitrite, S-nitrosothiols and nitrotyrosine

Low levels of exhaled S-nitrosothiols (naturally occurring bronchodilators) have been found in asthmatic children with respiratory failure. We have shown that S-nitrosothiols were reduced after 3 weeks of treatment with low-dose budesonide [37]. In contrast, there was a rapid and dose-dependent reduction in nitrite/nitrate in the same mildly asthmatic persons, suggesting that nitrite/nitrate is more sensitive to anti-inflammatory treatment. Elevated levels of nitrite/nitrate and nitrotyrosine have been found in exhaled condensate [80] and sputum [81] of patients with CF during both stable periods and exacerbations. In children with CF and normal lung function, however, the nitrite/nitrate concentrations in bronchoalveolar lavage fluid are normal and concentrations of S-nitrosothiols are reduced [82]. It may be speculated that nitration of proteins by myeloperoxidase [81,83] may be an additional source of nitrotyrosine in patients with CF who have a very low NO production. This may also explain the significantly higher levels of nitrotyrosine in exhaled breath condensate in CF [80], implying that an absence of an increase in exhaled NO does not exclude the possibility that NO participates in airway inflammation, including that in CF.

Electrolytes

A deficiency in magnesium and an elevation in calcium concentrations in exhaled breath condensate have been reported in atopic asthma [84]. We found that elevated levels of sodium and chloride in exhaled condensates of patients with CF correlate with sweat test results and disease severity (Balint *et al.*, unpublished data). Recently, a strong negative correlation between sputum chloride concentrations and exhaled NO has been demonstrated in patients with PCD [56], suggesting that impairment of airway mucociliary clearance might be monitored by exhaled/nasal NO and exhaled chloride levels.

Proteins, cytokines, hydrogen ions and products of lipid peroxidation

Measurement and identification of proteins in exhaled condensate are still controversial. Higher concentrations of total protein in exhaled condensate have been found in young smokers than in nonsmokers, whereas the levels of interleukin-1β and tumour necrosis factor-α were no different [85]. We found that interleukin-8 levels in exhaled condensate were mildly elevated in stable CF, but were more than doubled in unstable CF patients as compared with normal individuals (Balint *et al.*, unpublished data). An acidic microenvironment upregulates NOS2, making NO release moderately pH dependent [86], and a low pH of exhaled condensate has been reported in patients with acute asthma [23•].

Exhaled temperature

Measurements of humidity and exhaled temperature have been used to assess the conditioning function of the respiratory apparatus in asthma, COPD, pneumonia and pneumoconiosis [87,88]. We found that exhaled temperature measured under controlled conditions (standardized expiratory flow and pressure) [89] is low in CF and COPD [90], but is elevated in asthma [91,92]. Exhaled breath temperature may serve as a nonspecific, simple and inexpensive method for home monitoring of several upper and lower respiratory conditions, such as asthma, COPD, CF and rhinitis, and for assessing the effects of anti-inflammatory treatments.

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Conclusion

Accurate assessment of airway inflammation and oxidative stress, its severity and location within the lung is important to the clinical management of a variety of pulmonary conditions. It may allow the clinician to monitor the progression of the disease and to assess the efficacy of anti-inflammatory or antioxidant treatment. Breath analysis is currently a research procedure, but there is increasing evidence that it may have an important place in the diagnosis and management of lung disease in the future [1•]. This will drive the development of cheaper and more convenient analysers, which can be used in a hospital and later in a family practice setting; in turn, this will eventually lead to the development of personal monitoring devices for use by patients.

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